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The concept to be tested herein is that the various tyrosine kinase pathways known to contribute to anti-androgen resistance likely converge on a common signaling molecule that integrates the upstream signaling input and leads to androgen receptor activation in an androgen-independent manner. Identification of the signal-integrating molecule(s) would provide a valuable therapeutic target for prostate cancer. We hypothesized that the intracellular docking protein Cas, complexed with the adapter protein Crk, could have such a signal-integrating role. Our studies performed so far support a role for Crk in androgen resistance signaling pathways. At the same time, our studies suggest that a docking molecule other than Cas, but possessing a similar molecular weight, functions to couple Crk to the upstream tyrosine kinases. Thus, we have established a correlation between androgen independence and Crk signaling, as proposed in the aim 1 of our application. Our next objective is to molecularly identify the signal integrating docking molecule, and then proceed to aim 2 and examine whether activation of Crk signaling is causal to androgen independence in prostate cancer cells *in vitro*. Our long-term objective is to utilize the obtained information to develop specific and sensitive tools for diagnosis and therapeutics of anti-androgen resistance.

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Annual Report for W81XWH-04-1-0032

TITLE: MOLECULAR MECHANISMS OF HORMONE-REFRACTORY PROSTATE CANCER

1. Introduction

Prostate cancer begins as an androgen-dependent tumor that undergoes clinical regression in response to surgical or pharmacological strategies that reduce testosterone levels. A significant clinical problem in the treatment of prostate cancer is the conversion of androgen-sensitive tumors to a hormone-refractory state following anti-androgen therapy. Consequently, the majority of prostate cancer patients die of disseminated disease which is refractory to conventional therapies.

The molecular basis for androgen independence is poorly understood. It is known that anti-androgen therapies do not usually eliminate the expression of the androgen receptor (AR), and androgen insensitivity is therefore thought to be caused via ligand-independent activation of the AR (1). In support of this, it has been shown that a number of receptor tyrosine kinases can activate the AR in an androgen-depleted environment (2-5). In addition to peptide growth factors that bind to receptor tyrosine kinases, neuropeptides such as bombesin and neurotensin have also been implicated in anti-androgen resistance. Importantly, advanced prostate cancers often have increased numbers of neuroendocrine cells, and androgen independence is correlated with elevated levels of neuroendocrine markers in serum (6,7). Neuropeptides have been shown to induce androgen independence by activating the non-receptor tyrosine kinases Bmx, Src and Focal Adhesion Kinase (FAK) (8). Most notably, activation of Bmx, expression of which is known to be upregulated in advanced prostate carcinomas (9), was found to be a significant requirement for neuropeptide-induced androgen independence.

The underlying premise in our application is that the various receptor and non-receptor tyrosine kinases in prostate cancer cells likely converge on a common signaling molecule that integrates the upstream signaling input and leads to AR activation in an androgen-independent manner. Thus, we outlined a **concept** for our application that an ultimate common pathway would plausibly explain how so many seemingly disparate signals can contribute to androgen independence. As a corollary, identification of the signal-integrating molecule(s) in the pathway would provide a valuable therapeutic target for prostate cancer treatment. Based on several different criteria outlined in the original application, we postulated that tyrosine phosphorylation of the docking protein p130^{Cas} (Cas) has such a signal-integrating role in the development of hormone-refractory prostate cancer. Among these criteria was our then-unpublished finding that Cas is the main protein target for the kinase activity of Bmx. These studies have now been published (10), and the paper is included in the Appendix. As such, our objective within the Exploration-Hypothesis Development Award is to test the concept outlined above, and, at the same time, acquire preliminary data needed to allow the formulation of the hypothesis that Cas has a crucial and causal role in androgen independence in prostate cancer.

2. Body of the Report

In order to meet the objectives outlined above, two specific aims were set forth in our application. In the first aim, our goal was to examine whether activation of Cas signaling *correlates* with androgen independence in prostate cancer cells *in vitro*. If these studies were encouraging, we would proceed to aim 2 and examine whether activation of Cas signaling is *causal* to androgen independence in prostate cancer cells *in vitro*, and also *in vivo*.

During the first year of funding, we have focused our efforts on the first aim of the original application. Tasks that were to be accomplished as part of this aim are as follows:

- Task 1.** Examine Cas phosphorylation and Cas/Crk complex formation in neuropeptide-treated prostate cancer cells *in vitro* (months 1-6). These studies will answer the question whether activation of Cas signaling *correlates* with androgen independence in prostate cancer cells *in vitro*.
- Establish cell culture conditions for androgen-dependent LNCaP cells.
 - Examine the growth profile of androgen-depleted LNCaP cells upon stimulation with the synthetic androgen R1881, the neuropeptide bombesin, and control vehicle by an MTT assay.
 - Examine Cas and Crk expression levels and profiles in steady-state and differentially stimulated LNCaP cells by an immunoblot analysis.
 - Examine dose- and time-dependency, and intensity of Cas tyrosine phosphorylation in steady-state and differentially stimulated LNCaP cells by immunoprecipitating Cas with anti-Cas antibodies followed by a quantitative immunoblot analysis with anti-phosphotyrosine antibodies.
 - Examine dose- and time-dependency, and intensity of Cas/Crk interaction in steady-state and differentially stimulated LNCaP cells by immunoprecipitating Cas with anti-Cas antibodies followed by a quantitative immunoblot analysis with anti-Crk antibodies.
 - Examine the same in other *in vitro* culture models of androgen independence).

All studies outlined in Task 1 were accomplished during the first 6 months of this grant period. As such, we have, for example, established a culture model for androgen-dependent and -independent prostate cancer cells. While all experiments were technically successful, we obtained scientifically negative results on two key experiments. First, while Cas is expressed in LNCaP cells (as previously reported by us and others, see e.g. (11)), we failed to detect an enhanced Cas phosphorylation in LNCaP cells upon stimulation. Second, and as a corollary, we failed to see an enhanced Cas/Crk complex formation. Crk is an adapter protein that binds through its SH2-domain to tyrosine-phosphorylated docking proteins, such as Cas (12). Thus, the lack of enhanced Cas/Crk complex formation is explained by the lack of enhanced Cas phosphorylation in stimulated LNCaP cells.

As noted above, Crk has the potential to interact with other tyrosine-phosphorylated proteins than Cas. Thus, an interesting possibility exists that not Cas, but another docking protein (such as closely related Cas family members HEF-1/Cas-L or Sin/Efs, see (13)) could couple upstream tyrosine kinases to Crk signaling pathways in prostate cancer cells. We therefore extended our studies to examine this intriguing possibility. In these studies, LNCaP cells were plated in medium supplemented with 10% charcoal-stripped FBS and with synthetic androgen R1881 (4 nM), with bombesin (200 nM), or with DMSO (as a control). Cell lysates were subjected to immunoprecipitation experiments with anti-Crk antibodies, as well as affinity isolation experiments with a GST-fusion protein containing the sequences coding for the SH2-domain of Crk (see (14) for technical details). The precipitates were then analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibodies to detect any tyrosine-phosphorylated proteins bound to Crk in a stimuli-dependent (or -enhanced) manner. As shown in Figure 1 below, Crk becomes associated with tyrosine-phosphorylated protein(s) around 110-130 kDa in molecular weight in stimulated prostate cancer cells. While this corresponds to the molecular weight of Cas, immunoblotting with anti-Cas antibodies confirmed our earlier finding of lack of enhanced Cas/Crk complex formation upon stimulation of prostate cancer cells (not shown).

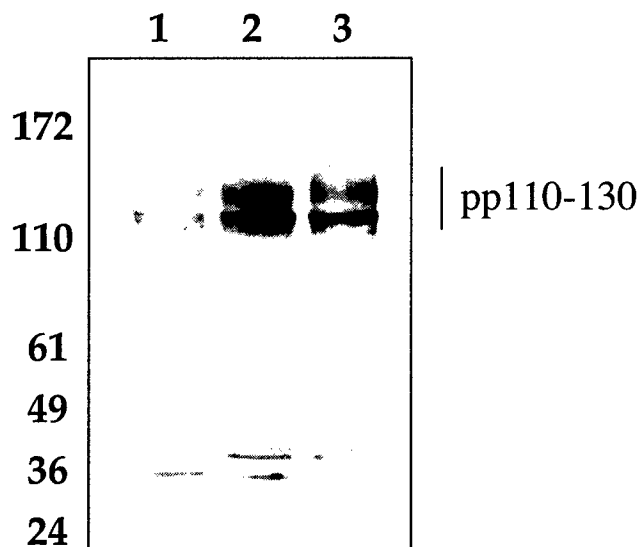


Figure 1. LNCaP cells were stimulated as described in the text, and cell lysates were immunoprecipitated with anti-Crk antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies. Lane 1, DMSO; lane 2, R1881; lane 3, bombesin.

Several known Crk-interacting molecules have a molecular weight in the range of 110-130 kDa and are known to be phosphorylated upon various stimuli. These include such signaling molecules as Cas-family member Hef-1/Cas-L, Gab1, Gab2, FAK, Cbl and C3G (see e.g. (12)). Our next task is to determine, by reciprocal immunoprecipitation experiments, which of these known binding partners might interact with Crk in prostate cancer cells. A mass spectrometry/proteomics approach will be undertaken to identify the protein(s) by means of mass fingerprinting and/or peptide sequencing, as deemed necessary. After this step, which we are confident will be highly feasible to achieve based on past experience and expertise, we will proceed to studies in aim 2. In our original application, aim 2 was outlined as to “examine whether activation of Cas signaling is *causal* to androgen independence in prostate cancer cells *in vitro*”. There will be no conceptual change to this Aim; rather, dominant-negative and constitutively active constructs of Crk described in the application will be utilized in this aim to evaluate the functional (cause-effect) role of Crk in androgen independence. We also proposed to utilize a form of Cas that lacks the tyrosine-phosphorylatable SD-domain and is incapable of interacting with Crk in these studies as an additional dominant-negative molecule. Use of this construct will be replaced with the corresponding dominant-negative mutant of another Crk-binding partner to be identified in the studies above. Of note, we already have such constructs for Hef-1, Gab1, Gab2 and Cbl at hand, and we therefore do not expect any delay in completing the studies outlined in the original application in a timely manner.

3. Key Research Accomplishments

- establishment of an *in vitro* prostate cancer model in the laboratory to examine androgen-independent signaling and growth of prostate cancer cells
- exclusion of Cas as a significant intermediary docking molecule in androgen-signaling pathways, at least in the LNCaP model system
- finding that the adapter protein Crk connects to additional upstream docking molecules in stimulated LNCaP cells

formation of a strong basis to expand studies to identify the upstream docking molecule(s), and its role, together with Crk, in androgen independence in prostate cancer cells

4. Reportable Outcomes

establishment of an in vitro prostate cancer model in the laboratory to examine androgen-independent signaling and growth of prostate cancer cells

Abassi, Y. A., Rehn, M., Ekman, N., Alitalo, K. & Vuori, K. (2003) p130^{Cas} couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. *J. Biol. Chem.* 278:35636-35643. This paper, published after the submission of the original application, provides validation of some of the preliminary data presented in the application.

4. Conclusions

Our data supports a role for a Crk-mediated signaling pathway in prostate cancer cells. Our original hypothesis identified p130Cas as a candidate tyrosine-phosphorylated docking protein that would connect upstream tyrosine kinases to the Crk pathway. Our studies described above, however, suggest that another docking protein of similar molecular weight, probably in a cancer tissue-specific manner, likely functions as such a signaling integrator. Immediate next studies have been planned to identify this molecule, and its function in androgen-independence. Conceptually, there is no change to the proposed tasks of the application. Minor changes in the technical design will be made as outlined above to successfully accomplish the goals of the application.

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6. Appendix

Abassi, Y. A., Rehn, M., Ekman, N., Alitalo, K. & Vuori, K. (2003) p130^{Cas} couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. *J. Biol. Chem*. 278:35636-35643.

p130^{Cas} Couples the Tyrosine Kinase Bmx/Etk with Regulation of the Actin Cytoskeleton and Cell Migration*

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Bmx/Etk, a member of the Tec/Btk family of nonreceptor kinases, has recently been shown to mediate cell motility in signaling pathways that become activated upon integrin-mediated cell adhesion (Chen, R., Kim, O., Li, M., Xiong, X., Guan, J. L., Kung, H. J., Chen, H., Shimizu, Y., and Qiu, Y. (2001) *Nat Cell Biol.* 3, 439–444). The molecular mechanisms of Bmx-induced cell motility have so far remained unknown. Previous studies by us and others have demonstrated that a complex formation between the docking protein p130^{Cas} (Cas) and the adapter protein Crk is instrumental in connecting several stimuli to the regulation of actin cytoskeleton and cell motility. We demonstrate here that expression of Bmx leads to an interaction between Bmx and Cas at membrane ruffles, which are sites of active actin remodeling in motile cells. Expression of Bmx also enhances tyrosine phosphorylation of Cas and Cas-Crk complex formation, and coexpression of Bmx with Cas results in an enhanced membrane ruffling and haptotactic cell migration. Importantly, a mutant form of Bmx that fails to interact with Cas also fails to induce cell migration. Furthermore, expression of a dominant-negative form of Cas that is incapable of interacting with Crk inhibits Bmx-induced membrane ruffling and cell migration. These studies suggest that Bmx-Cas interaction, phosphorylation of Cas by Bmx, and subsequent Cas-Crk complex formation functionally couple Bmx to the regulation of actin cytoskeleton and cell motility.

Cytoskeletal dynamics and cell migration are critical aspects of normal development, wound healing, and inflammatory response. Integrins, which are the major cell surface receptors that interact with extracellular matrix components, generate signals that regulate reorganization of actin cytoskeleton and cell motility in coordination with signaling events stimulated by receptors for soluble growth factors (1, 2). Focal adhesion kinase (FAK)¹ has emerged as a crucial molecule in integrating

signals that lead to the regulation of cell motility (reviewed in Refs. 3 and 4). Ligand binding of integrins results in catalytic activation of FAK and in its autophosphorylation at Tyr-397, which serves as a binding site for several Src homology 2 (SH2) domain-containing proteins including Src kinases. The binding of Src kinases to FAK leads to further phosphorylation of FAK and also to phosphorylation and activation of a number of cytoskeleton-linked proteins, which transduce integrin-generated signals to downstream signaling pathways (reviewed in Refs. 3–6). The most compelling evidence regarding the role of FAK in cell migration stems from the observation that FAK knock-out cells are defective in both haptotactic and chemotactic cell migration (7–11). Exogenous expression of FAK in turn is known to enhance cell motility of various cell types, and a number of groups have reported that FAK protein levels and/or its activity are up-regulated in invasive cancer cells (see Ref. 12 and references therein).

FAK is thought to mediate cell migration by recruitment and phosphorylation of the docking protein p130^{Cas} (Cas). Thus, a dominant-negative form of Cas has been shown to inhibit FAK-mediated haptotactic cell migration (13). Cas contains multiple protein-protein interaction domains, including an SH3 domain at the N terminus, a Src-binding (SB) domain toward the C terminus, and an interior substrate domain (SD) (see Fig. 3A) (14). The SH3 domain of Cas is thought to regulate the tyrosine phosphorylation status of Cas because it interacts with tyrosine kinases, such as FAK (15, 16) and its homologue Pyk2 (17, 18), and with tyrosine phosphatases such as PTP1B (19) and PTP-PEST (20). The SB domain is a second region of Cas interaction with tyrosine kinases and contains a proline-rich motif that can interact with the SH3 domains of Src family kinases and a tyrosine phosphorylation site that can bind to the SH2 domains of Src kinases (21). Both FAK and PYK2 as well as Src family kinases have been implicated in integrin-dependent phosphorylation of Cas (22–28). The SD domain of Cas is the major region of tyrosine phosphorylation and consists of 15 potential tyrosine phosphorylation sites (YXXP). When phosphorylated, the YXXP motif conforms to the binding consensus for the Crk SH2 domain (29) and integrin-dependent tyrosine phosphorylation of Cas is known to lead to recruitment of the adapter protein c-CrkII (22). The Cas-Crk complex has been shown to serve as a “molecular switch” for induction of cytoskeletal dynamics and cell migration. Thus, overexpression of wild-type Cas alone or in combination with Crk leads to an enhancement of membrane ruffling and haptotactic cell migration on extracellular matrix, whereas dominant-negative forms of Cas and Crk that are unable to interact with each other block stimuli-induced cell motility (30, 31). The most direct role for Cas in regulating cytoskeletal dynamics comes from studies done with fibroblasts derived from Cas^{−/−} embryos. These fibroblasts contain disorganized actin filaments, deficiency in cell spreading, and decreased haptotaxis toward the integrin

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¹ The abbreviations used are: FAK, focal adhesion kinase; SH, Src homology; Cas, docking protein p130^{Cas}; SB, Src-binding; FN, fibronectin; PLL, poly-L-lysine; PBS, phosphate-buffered saline; PAE, porcine aortic endothelial; GST, glutathione S-transferase; HA, hemagglutinin; WT, wild type; KD, kinase dead; SD, substrate domain; pY, anti-phosphotyrosine.

ligand fibronectin (FN), very similar to FAK and Src-deficient fibroblasts (7, 27, 32–36).

It was shown recently that integrins regulate the activity of another tyrosine kinase, ETK/Bmx, which is a member of the Tec/Btk family of tyrosine kinases (37). In addition to the SH2, SH3, and kinase domains, the Tec family of tyrosine kinases also contain an N-terminal pleckstrin homology domain (38, 39). Chen *et al.* (37) reported that adhesion of endothelial cells on FN induced tyrosine phosphorylation of Bmx, which correlated with its interaction with FAK. The interaction between Bmx and FAK was found to occur via the pleckstrin homology and FERM domains, respectively. Bmx mutants that failed to interact with FAK were found to inhibit FAK- and FN-induced cell migration, indicating that integrin-dependent Bmx-FAK interaction is required for haptotactic cell motility by an unknown mechanism.

In this report, we have investigated the possibility that Cas would function as a downstream target for Bmx and mediate Bmx-induced cell motility. We found that expression of Bmx leads to a complex formation between Bmx and Cas at membrane ruffles, which are sites of active actin remodeling in motile cells. Our data also indicate that Bmx enhances tyrosine phosphorylation of Cas and its interaction with Crk. Furthermore, Bmx and Cas were found to enhance membrane ruffling and haptotactic cell migration and Bmx-induced membrane ruffling and cell migration were found to take place in a Cas-dependent manner. These studies suggest that phosphorylation of Cas by Bmx functionally couples Bmx to the regulation of actin cytoskeleton and cell motility.

MATERIALS AND METHODS

Constructs and Reagents—The pCIneo expression vectors for HA-tagged forms of wild-type and kinase-dead Bmx have been described previously (40, 41). The HA-tagged Bmx SH2 mutant (R322V) in pCIneo was generated by using the QuikChange site-directed mutagenesis kit (Stratagene). Wild-type Cas and its Δ SD and Δ SB mutant forms in the pEBG mammalian expression vector have been described previously (30, 42). The pEBG-Cas SH3 mutant (W137A) was generated by site-directed mutagenesis as described above. Expressed proteins in the pEBG-vector contain an N-terminal GST tag. The GST-CasSD, GST-CasSB, and GST-CasSH3 constructs in the pGEX-3X bacterial expression vector coding for the GST fusion proteins of the SD, SB, and SH3 domains of Cas, respectively (14), were obtained from Dr. Hisamaru Hirai.

The anti-Cas, anti-Bmx, and anti-Crk monoclonal antibodies and the horseradish peroxidase-conjugated anti-phosphotyrosine antibody PY-20 were purchased from Transduction Laboratories. The anti-Cas polyclonal (C-20 and N-17) and anti-HA monoclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-Src monoclonal antibody 327 and the anti-GST polyclonal antibody were generous gifts from Drs. Joan Brugge (Harvard Medical School) and Elena Pasquale (The Burnham Institute), respectively. Rhodamine-phalloidin as well as Alexa 458- and Alexa 546-labeled secondary antibodies were from Molecular Probes.

Cell Culture and Transfections—COS-7 cells and 293T cells were maintained in Dulbecco's modified Eagle's high glucose medium (Irvine Scientific) containing 10% fetal bovine serum at 37 °C and 5% CO₂. Porcine aortic endothelial (PAE) cells were maintained in Ham's F12 growth medium (Invitrogen) containing 10% fetal bovine serum. COS-7 and 293T cells were transfected with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's protocols.

Immunoprecipitation, Immunoblotting, and Kinase Assays—COS-7 and 293T cells transfected with the various expression plasmids were washed in PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 1% Triton X-100, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were immunoprecipitated with anti-HA or anti-GST antibodies, and the immunoprecipitates were captured with protein A-Sepharose, washed, and eluted with 2 \times sample buffer. The immunoprecipitates were loaded on 4–12% SDS-polyacrylamide gels (Novex) and immunoblotted with the indicated antibodies. For coimmunoprecipitation of endogenous proteins, PAE cells were serum-starved for 16 h, detached, held in suspension for 2 h, and replated on FN-coated

(10 μ g/ml) or poly-L-lysine (PLL)-coated (20 μ g/ml) plastic dishes for 30 min. The cells were washed in PBS and lysed in modified radioimmune precipitation assay buffer as described in Chen *et al.* (37) and immunoprecipitated with anti-Bmx antibodies followed by immunoblotting with anti-Cas antibodies.

In vitro immunocomplex kinase assays were carried out by immunoprecipitating HA-tagged Bmx or c-Src from COS-7 cell lysates as described above. The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM Hepes, pH 7.4, and 10 mM MgCl₂). The immunoprecipitates were equally divided and resuspended in 18 μ l of kinase buffer with or without 1 μ M Src inhibitor SU6656 in the presence of either 2 μ g of GST or 2 μ g of GST-CasSD as a putative substrate. After a 15-min incubation at 30 °C, the reactions were initiated by the addition of 10 μ M ATP and 10 μ Ci of [³²P]ATP (final concentrations) and the reaction was allowed to proceed at 30 °C for an additional 20 min. The reactions were terminated by the addition of 20 μ l of 2 \times sample buffer, and the samples were heated at 95 °C for 5 min and fractionated on an SDS-PAGE. The gel was washed, stained, destained, dried, and subjected to autoradiography for 12 h.

GST Fusion Protein Expression—DH5 α cells harboring the various GST-Cas constructs (see above) were grown overnight, diluted 5-fold with fresh LB medium for 2 h, and induced with isopropyl-1-thio- β -D-galactopyranoside for an additional 2 h. The fusion proteins were purified as described previously (43). To obtain tyrosine-phosphorylated GST-CasSD and GST-CasSB fusion proteins, the pGEX-CasSD and the pGEX-CasSB plasmids were transformed into *Escherichia coli* TKX1-competent cells (Stratagene), which harbor a plasmid-encoded, inducible tyrosine kinase gene. The cells were grown, induced, and lysed according to the manufacturer's protocols.

Cell Migration Assay—Haptotactic cell migration assays in COS-7 cells were performed as described previously (44). The underside of the Transwell membranes was coated with 10 μ g/ml FN for 2 h at 37 °C. 8.0 \times 10⁵ COS-7 cells in 10-cm dishes were transfected with 0.5 μ g of the indicated plasmids along with 0.5 μ g of pEGFP plasmid as a marker for the transfected cells. The cells were detached with trypsin-EDTA, washed in serum-free medium, 0.5% bovine serum albumin, and counted and adjusted to 10⁶ cells/ml. 100 μ l of the cell suspension were placed in the Transwell membranes and allowed to migrate to the underside for 3 h at 37 °C. The cells on the top chamber were removed with a cotton swab, and the cells migrating to the underside of the filter were fixed in 4% paraformaldehyde. The filters were cut and mounted on a coverslip, and the green fluorescent protein-positive cells were visualized and photographed using a Nikon immunofluorescence microscope. As a control for transfection efficiency and cell spreading, 5.0 \times 10⁵ cells were allowed to spread on FN-coated chamber slides for 30 min and then fixed and visualized under the microscope.

Immunofluorescence Microscopy—PAE or COS-7 cells that had been transfected with the indicated constructs were serum-starved for 16 h, detached, and resuspended in serum-free medium containing 0.25% bovine serum albumin. The cells were kept in suspension for 2 h and then seeded on PLL- or FN-coated coverslips for 30 min. The cells were washed in PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in PBS. The cells were blocked with 0.5% bovine serum albumin and 5% goat serum in PBS followed by incubation with the indicated antibodies and rhodamine-phalloidin. The cells were visualized and photographed as described previously (44).

RESULTS

Bmx Induces Tyrosine Phosphorylation of Cas and Cas-Crk Interaction—The tyrosine kinase Bmx is activated in response to cell adhesion on FN and is required for cell migration on FN (37). The docking protein Cas becomes tyrosine-phosphorylated in an adhesion-dependent manner on FN and other extracellular matrix components and is involved in maintaining the integrity of the actin cytoskeleton and regulating cell migration by recruiting the adapter protein Crk (22, 30, 31, 45–48). To investigate the putative role of Cas in Bmx-induced cell motility, we first sought to determine whether Bmx can induce tyrosine phosphorylation of Cas and a subsequent interaction between Cas and Crk. COS-7 cells were transfected with expression vectors coding for GST-tagged wild-type Cas alone or together with HA-tagged wild-type Bmx (HA-Bmx WT) or with HA-tagged kinase-dead Bmx (HA-Bmx KD). Forty-eight hours after transfection, the cells were lysed, immunoprecipitated with anti-GST antibodies, and immunoblotted with anti-phos-

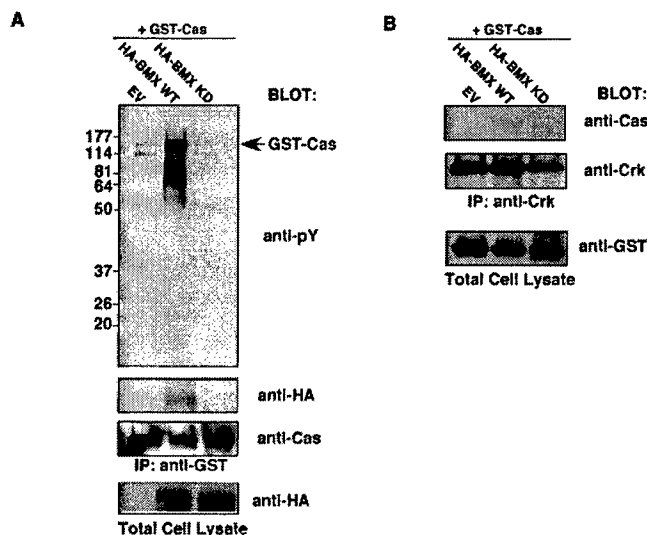


FIG. 1. Bmx induces tyrosine-phosphorylation of Cas and Cas/Crk complex formation. A, cell lysates prepared from COS-7 cells transfected with empty vector (EV), HA-Bmx WT, or HA-Bmx KD plasmids together with plasmids coding for GST-Cas WT were immunoprecipitated with anti-GST antibodies. The immunoprecipitates were immunoblotted with anti-phosphotyrosine (pY) antibodies (upper panel), and the membrane was subsequently stripped and reprobed with anti-HA and anti-Cas antibodies as indicated. Total cell lysates were probed with an anti-HA antibody to determine the expression levels of the HA-Bmx WT and HA-Bmx KD proteins. B, similarly prepared lysates were immunoprecipitated with anti-Crk antibodies followed by immunoblotting with anti-Cas and anti-Crk antibodies. Total cell lysates were probed with anti-GST antibodies to confirm equal levels of Cas proteins in the lysates.

phosphotyrosine antibodies. When expressed alone, GST-Cas was only weakly tyrosine-phosphorylated (Fig. 1A). Coexpression of HA-Bmx WT in turn led to a significant increase in Cas tyrosine phosphorylation. Expression of HA-Bmx KD had no effect on Cas phosphorylation, indicating that the observed increase in Cas tyrosine phosphorylation was dependent on a functional kinase domain in Bmx.

To examine the possibility that Cas and Bmx interact in cells, the anti-GST immunoprecipitates were probed with anti-HA antibodies. As shown in Fig. 1A, HA-Bmx WT but not HA-Bmx KD coimmunoprecipitated with GST-Cas, suggesting that Bmx forms a complex with Cas in a manner that depends on the kinase activity of Bmx and/or on the tyrosine phosphorylation status of Cas. The amount of Bmx interacting with Cas was approximately 5–20% of the total amount of Bmx, slightly varying from one experiment to another.

To determine whether Bmx modulates the interaction between Cas and Crk, the cellular lysates described above were immunoprecipitated with anti-Crk antibodies followed by immunoblotting with anti-Cas antibodies. As shown in Fig. 1B, coexpression of Cas with HA-Bmx WT but not with HA-Bmx KD induced an interaction between Crk and Cas. Taken together, expression of Bmx leads to an interaction between Bmx and Cas and to an enhanced tyrosine phosphorylation of Cas and Cas/Crk complex formation.

Integrin-dependent Localization and Interaction of Endogenous Bmx and Cas Proteins in Endothelial Cells—Integrin-mediated cell adhesion and spreading lead to an increase in Cas phosphorylation and localization of Cas to the cell periphery in COS-7 cells (30). Bmx in turn has been shown to localize to the perimeter of FN-plated endothelial cells (37). Therefore, we examined the subcellular localization of endogenous Cas and Bmx proteins in endothelial cells upon integrin ligand binding and the capability of integrin ligation to modulate

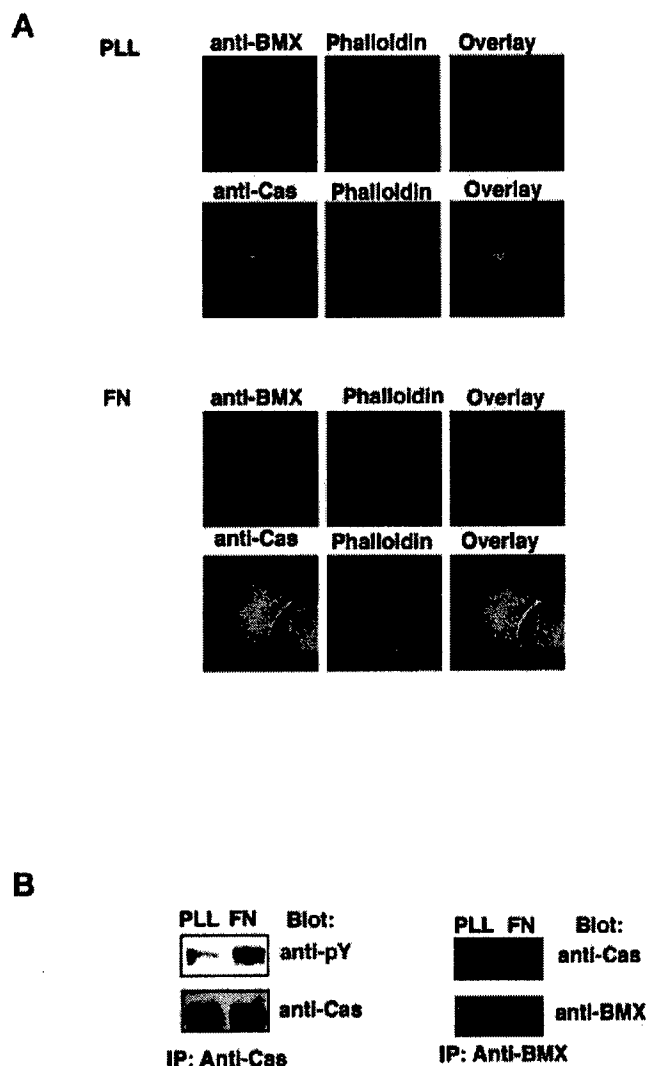


FIG. 2. Cas and Bmx localize to the cell periphery in endothelial cells plated on FN and biochemically interact upon integrin-mediated cell adhesion. A, PAE cells were serum-starved, detached, and seeded on PLL or FN-coated coverslips for 30 min. The cells were fixed in 4% paraformaldehyde, permeabilized, and stained with anti-Bmx or anti-Cas primary antibodies followed by Alexa 458 secondary antibody staining. The cells were costained with rhodamine-phalloidin to visualize the actin cytoskeleton. B, serum-starved PAE cells were detached and replated on PLL- or FN-coated dishes for 30 min. The cells were lysed in RIPA buffer and immunoprecipitated with anti-Cas antibodies (left panels) followed by immunoblotting with pY antibodies. The blot was stripped and reprobed with anti-Cas antibodies to assess the levels of the immunoprecipitated Cas. The lysates were also precipitated with anti-Bmx antibodies (right panels) followed by immunoblotting with anti-Cas antibodies. The blot was stripped and reprobed with anti-Bmx antibodies to confirm equal loading.

Cas-Bmx interaction. To this end, serum-starved PAE cells were detached and replated on FN-coated coverslips for 30 min. As a control, the cells were also plated on PLL to which they adhered in an integrin-independent manner. The cells were fixed and stained with monoclonal anti-Cas and anti-Bmx antibodies as well as with rhodamine-phalloidin to visualize the actin cytoskeleton. As shown in Fig. 2A, PAE cells failed to spread on PLL-coated surfaces and exhibited a diffuse Cas and Bmx staining on PLL. In contrast, Bmx and Cas localized to the perimeter of the spreading cells on FN and this localization was coincident with the staining of cortical actin by rhodamine-phalloidin. This staining pattern was suggestive of colocalization of Bmx and Cas in the cell periphery upon integrin-mediated cell adhesion. Unfortunately, our efforts to study this

further by coimmunostaining with anti-Cas and anti-Bmx antibodies were hampered by the low sensitivity of polyclonal anti-Cas antibodies in immunofluorescence and by unspecific reactivity of the monoclonal anti-Bmx antibodies with secondary rabbit antibodies (data not shown). Biochemical means were therefore used to examine whether endogenous Cas and Bmx proteins physically interact in endothelial cells and whether the interaction is modulated by integrin ligation. To this end, serum-starved PAE cells were detached and plated on PLL- or on FN-coated plates for 30 min. The cells were lysed and immunoprecipitated with anti-Cas and anti-Bmx antibodies. Immunoblot of Cas immunoprecipitates with anti-phosphotyrosine antibodies indicated that Cas tyrosine phosphorylation was significantly augmented, as expected, upon cell adhesion on FN but not on PLL. Importantly, the enhanced phosphorylation of Cas correlated with the interaction of Cas with Bmx (Fig. 2B). These results indicate that integrin-mediated cell adhesion leads to the localization of endogenous Cas and Bmx proteins at the cell periphery and to an enhanced interaction between the two proteins as assessed by coimmunoprecipitation studies.

Bmx Induces Tyrosine Phosphorylation of the Cas SD *in Vivo* and *in Vitro*—To further investigate the molecular mechanisms of Bmx-Cas interaction, HA-Bmx WT was coexpressed with various mutant forms of GST-tagged Cas in COS-7 cells and the tyrosine phosphorylation status of the expressed Cas proteins and their interaction with Bmx were analyzed. When expressed alone, wild-type GST-Cas and the various mutant forms of Cas exhibited little tyrosine phosphorylation (Fig. 3B). Upon coexpression with Bmx, wild-type Cas and a form of Cas that lacked a functional SH3 domain became robustly tyrosine-phosphorylated (Fig. 3B). These two forms of Cas also readily interacted with Bmx (Fig. 3C). The Cas-SH3 mutant protein is incapable of interacting with FAK (data not shown) (16), suggesting that Cas interaction with and phosphorylation by Bmx do not require a direct interaction between Cas and FAK.

A form of Cas in which the SD containing 15 potential tyrosine phosphorylation sites had been deleted (Cas Δ SD) failed to demonstrate any tyrosine phosphorylation upon Bmx coexpression (Fig. 3B), despite the fact that this mutant interacted with Bmx to the same extent as the wild-type form of Cas (Fig. 3C). Therefore, these results suggest that the SD domain of Cas may be a target for the kinase activity of Bmx. In support of this, immunoprecipitated wild-type Bmx readily phosphorylated recombinantly expressed SD domain of Cas in an *in vitro* immunocomplex kinase assay (Fig. 3D). Importantly, the Src inhibitor SU6656 had no effect on Bmx-mediated phosphorylation of the SD domain *in vitro*, although it completely inhibited phosphorylation of CasSD by immunoprecipitated Src kinase. This finding suggests that the kinase activity of Bmx itself, rather than that of a potentially coimmunoprecipitating Src, is responsible for tyrosine phosphorylation of CasSD *in vitro* and perhaps also *in vivo* (see "Discussion"). As mentioned earlier, the SD domain of Cas when tyrosine-phosphorylated binds to Crk and therefore this model is consistent with our observation in Fig. 1 that Bmx enhances an interaction between Cas and Crk *in vivo*.

The SH2 Domain of Bmx Interacts with the SB Domain of Cas in a Phosphorylation-dependent Manner and Is Required for Integrin-induced Bmx-Cas Interaction—As shown in Fig. 3, B and C, a form of Cas in which the SB domain had been deleted (Cas Δ SB) demonstrated a slightly reduced tyrosine phosphorylation and a greatly reduced interaction with Bmx compared with wild-type Cas. This finding is consistent with a model in which the SB domain of Cas is required for the bulk of Cas-Bmx interaction that can be observed under our lysis con-

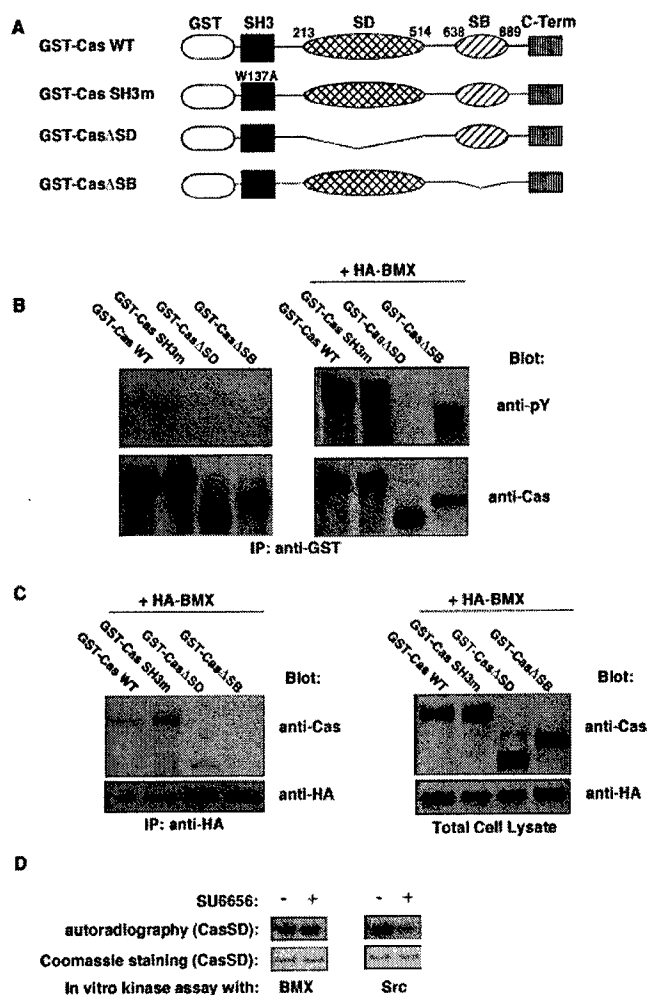


FIG. 3. Bmx induces phosphorylation of SD of Cas *in vitro* and *in vivo* and interacts with the SB domain of Cas *in vivo*. A, schematic presentation of the structure of Cas and the various constructs used in this study. The numbering of the amino acid corresponds to the amino acid sequence of rat Cas described in Ref. 14. B, COS-7 cells expressing GST-Cas WT, GST-Cas SH3m (W137A), GST-Cas Δ SD or GST-Cas Δ SB without (left panels) or with coexpressed HA-Bmx WT (right panels) were lysed and immunoprecipitated with anti-GST antibodies. The immunoprecipitates were subsequently immunoblotted with pY antibodies (upper panels). The membranes were stripped and reprobed with anti-Cas antibodies (lower panels) to confirm equal loading. C, cell lysates prepared from COS-7 cells expressing the various Cas-constructs described above together with HA-Bmx WT were immunoprecipitated with anti-HA antibodies (left panels) followed by immunoblotting with anti-Cas antibodies. The blot was stripped and reprobed with an anti-HA antibody. In the right panels, total cell lysates were immunoblotted with anti-Cas (upper panel) and anti-HA (lower panel) antibodies. D, COS-7 cells expressing HA-Bmx WT were lysed and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were subjected to an *in vitro* immunocomplex kinase assay using purified GST-CasSD as the substrate in the presence or absence of 1 μ M Src inhibitor SU6656 (66). As a control, endogenous Src was immunoprecipitated and subjected to an *in vitro* immunocomplex kinase assay in the presence or absence of the inhibitor. The kinase reactions were carried out as described under "Materials and Methods." GST-CasSD loading in the kinase reaction was examined by Coomassie Blue staining.

ditions. To further test this notion, *in vitro* pull-down experiments were carried out using bacterially produced, unphosphorylated or phosphorylated GST fusion proteins of the SH3, SD, or SB domains of Cas as bait. As shown in Fig. 4A, unphosphorylated forms of these fusion proteins failed to interact with HA-Bmx WT in COS-7 cell lysates. Phosphorylated form of the SB domain but not of the SD domain of Cas in turn readily

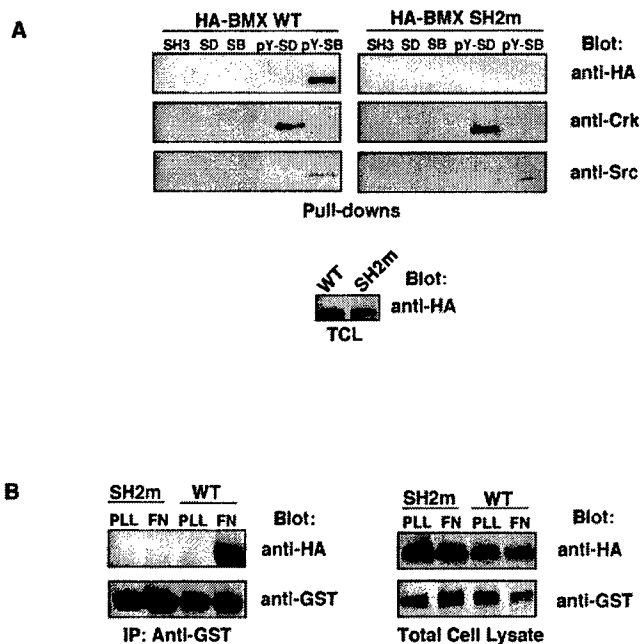


FIG. 4. The SH2 domain of Bmx interacts with the SB domain of Cas in a tyrosine phosphorylation-dependent manner *in vitro* and mediates integrin-induced Bmx-Cas interaction *in vivo*. *A*, COS-7 cells expressing HA-Bmx WT (left panels) or HA-Bmx SH2m (R322V) (right panels) were lysed and subjected to a pull-down assay by using GST-CasSH3, GST-CasSD, GST-CasSB, and GST-CasSD that had been phosphorylated on tyrosine residues (pY-SD) (see "Materials and Methods") or GST-CasSB phosphorylated on tyrosine residues (pY-SB) as a bait. The pull-down complexes were fractionated on SDS-PAGE and immunoblotted with anti-HA antibodies to assess the extent of Bmx WT and Bmx SH2m binding. The blots were stripped and reprobed with anti-Src and anti-Crk antibodies to assess the binding of Src and Crk to the various fusion proteins as a control. Ponceau S staining of the blots revealed that equal amounts of GST fusion proteins were used in the pull-down assays and anti-phosphotyrosine immunoblotting confirmed tyrosine phosphorylation of the pY-SD and pY-SB proteins but not of the other fusion proteins (data not shown). The bottom panel shows anti-HA immunoblot analysis of pull-down lysates (TCL) of COS-7 cells expressing HA-Bmx WT and HA-Bmx SH2m. *B*, COS-7 cells expressing HA-Bmx SH2m (R322V) and HA-Bmx WT together with GST-Cas WT were serum-starved, detached, and plated on PLL- or FN-coated dishes for 30 min. The cells were washed with PBS, lysed in lysis buffer, and immunoprecipitated with anti-GST antibodies. The immunoprecipitates were fractionated on SDS-PAGE and immunoblotted with anti-HA antibodies (left panel). Anti-HA and anti-GST immunoblotting of total cell lysates (right panels) were carried out to confirm equal expression levels.

precipitated Bmx. These studies suggested that similar to Src kinases, the SH2 domain of Bmx might recognize a tyrosine-phosphorylated motif in the SB domain of Cas. Indeed, we found that a form of Bmx in which the SH2 domain had been inactivated by a point mutation (HA-Bmx SH2m) failed to interact with phosphorylated SB domain of Cas. In control experiments, phosphorylated GST-CasSD protein pulled-down Crk and the phosphorylated GST-CasSB interacted with Src in the cell lysates as expected. Thus, our findings suggest that the SB domain of Cas, when phosphorylated, mediates the binding of Cas to the SH2 domain of Bmx.

To determine whether the SH2 domain of Bmx is required for integrin-induced Bmx-Cas interaction *in vivo*, COS-7 cells expressing GST-Cas together with HA-Bmx WT or HA-Bmx SH2m were detached and plated on PLL- or FN-coated dishes for 30 min. Cell lysates were subjected to immunoprecipitation with anti-GST antibodies followed by immunoblotting with anti-HA antibodies. As shown in Fig. 4B, wild-type Bmx readily interacted with Cas in an integrin ligand binding-dependent manner whereas Bmx SH2 mutant failed to do so. Taken to-

gether, these results demonstrate that the SH2 domain of Bmx binds to the tyrosine-phosphorylated CasSB domain *in vitro* and that this interaction is required for integrin-induced Bmx-Cas interaction *in vivo*.

Bmx Induces Actin Reorganization and Cell Migration in a Cas-dependent Manner—Bmx has been shown to enhance haptotactic cell migration on FN (37). Likewise, Cas augments haptotactic cell migration and induces membrane ruffling of fibroblast spreading on FN (30). We next determined whether Bmx also induces membrane ruffling of cells on FN and, if so, whether this activity is dependent on Cas. Serum-starved COS-7 cells expressing Bmx alone, Cas alone, Bmx with Cas, or Bmx with Cas Δ SD (this form of Cas functions as a dominant-negative with respect to Cas-dependent membrane ruffling and cell motility as it uncouples Cas from Crk (30)) were detached and replated on FN-coated coverslips for 30 min. The cells were fixed and stained with anti-HA and anti-Cas antibodies and with rhodamine-phalloidin as indicated in Fig. 5A. Expression of Bmx was found to significantly enhance membrane ruffling at the perimeter of the spreading cells as judged by phalloidin staining. As shown previously (30, 31, 48), expression of Cas in COS-7 cells also led to increased membrane ruffling. Furthermore, both Bmx and Cas localized to the sites of membrane ruffles, as assessed by colocalization with the filamentous actin at the cell periphery. Control-transfected cells displayed very little membrane ruffling (Fig. 5A), and the bulk of filamentous actin remained organized in stress fibers. Coexpression of Cas with Bmx led to a colocalization of the two proteins at the periphery of the cell and to an enhancement of lamellipodia formation. Importantly, coexpression of Cas Δ SD with Bmx resulted in a significant inhibition of Bmx-induced cell spreading and membrane ruffling, suggesting an important functional role for Cas in Bmx signaling.

Because coexpression of Cas and Bmx led to an enhanced membrane ruffling that is suggestive of a migratory phenotype, we next examined the effect of Bmx and Cas coexpression on haptotactic cell migration on FN. As shown in Fig. 5B, expression of either Bmx or Cas alone led to an ~2-fold increase in cell migration in COS-7 cells, whereas coexpression of Bmx with Cas further increased cell migration by ~3-fold over the base line. Coexpression of Cas Δ SD with Bmx inhibited Bmx-induced cell migration, correlating with Cas Δ SD-mediated inhibition of Bmx-induced membrane ruffling. Importantly, the SH2 mutant form of Bmx that failed to interact with Cas (Fig. 4B) failed to augment cell migration above background levels. Taken together, these data indicate that Bmx induces membrane ruffling, cell spreading, and haptotactic cell motility on FN. Coexpression of Cas with Bmx enhances the Bmx-induced membrane ruffling activity and cell migration, whereas a mutant form of Cas, which uncouples Cas from Bmx-induced tyrosine phosphorylation and subsequent Crk interaction, inhibits these cellular functions. Likewise, a mutant form of Bmx that fails to interact with Cas also fails to enhance haptotactic cell migration, further supporting a functional significance for Bmx-Cas interaction.

DISCUSSION

Increasing evidence suggest that Bmx and other members of the Tec/Btk family of nonreceptor tyrosine kinases play central but diverse modulatory roles in various signaling processes. Both genetic and biochemical data have elucidated the role of Tec kinases in the regulation of cell survival, growth, and differentiation in signal transduction by a number of different extracellular stimuli, which include activation of growth factor receptors, cytokine receptors, and antigen receptors (49). An emerging new role for Tec kinases and especially for Bmx is cytoskeletal organization and cell motility. In contrast to the

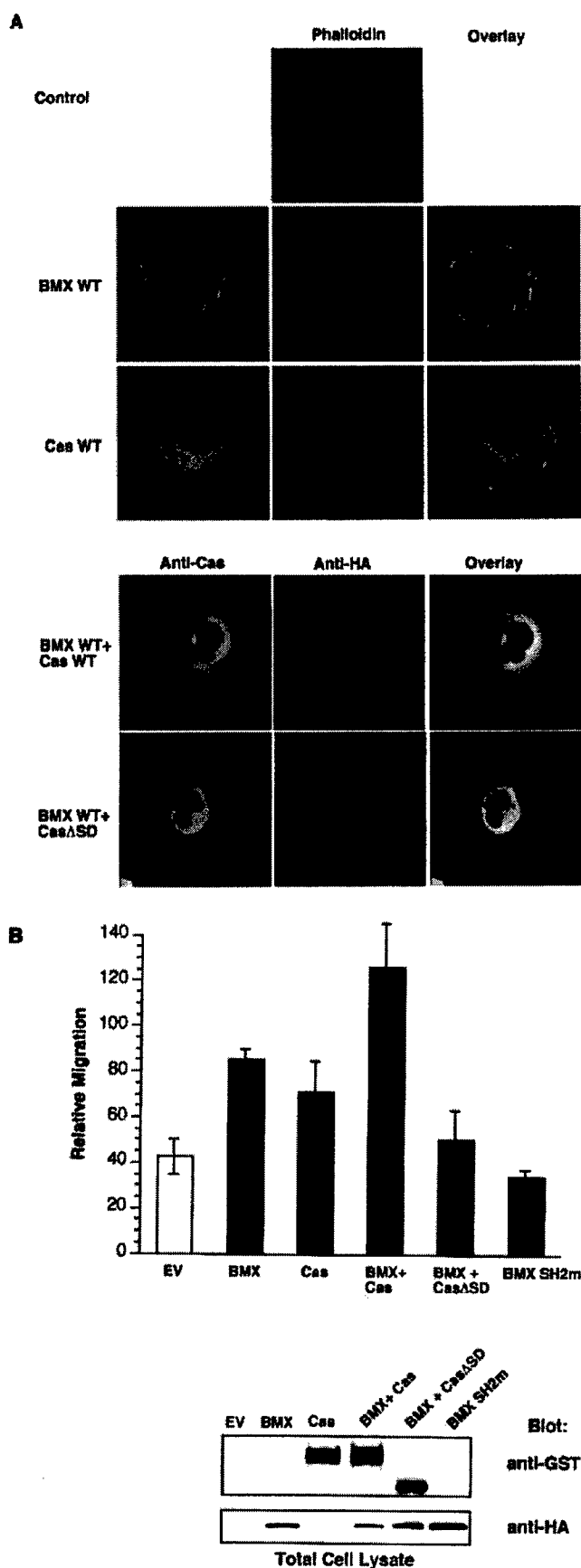


Fig. 5. Bmx and Cas enhance membrane ruffling and haptotactic cell migration. A, serum-starved COS-7 cells expressing HA-Bmx WT or GST-Cas WT or HA-Bmx WT together with GST-Cas WT or GST-CasASD were detached and replated on FN-coated coverslips for

hematopoietic cell-restricted expression of other members of the Tec family, Bmx is expressed in various other cell types with great migratory potential including endothelial cells, epithelial cells, and metastatic carcinoma cells (38, 50). Chen *et al.* (37) recently established that Bmx is involved in signaling downstream of integrins, which are key receptors regulating actin cytoskeleton and cell motility. These authors further demonstrated a causal relationship between Bmx expression and cell motility by showing that antisense-induced reduction of Bmx protein expression is associated with a significant inhibition of motility of prostate and breast carcinoma cells. Chen *et al.* (37) also showed that the activation of Bmx by integrins is mediated by FAK, a key mediator of integrin signaling events leading to modulation of cell motility, and that Bmx is essential for FAK-induced cell motility (37). The molecular mechanisms as to how Bmx exerts its effects on the cytoskeleton and cell motility downstream of FAK and integrin signaling pathways have remained unexplored. In this report, we identify the docking protein Cas as a target for Bmx activity and demonstrate that Cas is a mediator of Bmx-induced cell spreading and cell motility on FN. Therefore, our studies identify a potential molecular mechanism as to how Bmx connects to the regulation of actin cytoskeleton.

Our results demonstrate that integrin-mediated cell adhesion regulates a complex formation between Bmx and Cas and that this complex formation coincides with tyrosine phosphorylation of Cas and localization of Bmx and Cas in the edges of the spreading cell. Our studies with various mutant forms of Cas demonstrate that the complex formation is mediated by the SB domain of Cas, and the *in vitro* affinity experiments suggest that the SH2 domain of Bmx directly recognizes a phosphorylated tyrosine residue in CasSB. Thus, Bmx-Cas interaction probably requires a prior phosphorylation of Cas, which could be mediated by FAK and/or Src kinases in adherent cells (see Introduction). It remains to be determined whether Bmx, indirectly or directly, contributes to Cas phosphorylation at a site recognized by the Bmx-SH2 domain. This possibility is suggested by our finding that the kinase-dead form of Bmx fails to interact with Cas in adherent cells under our experimental conditions (see Fig. 1), although it is also possible that the SH2 domain of Bmx is unavailable for target binding in the context of an inactive kinase due to steric hindrance (49). Also, we cannot rule out the possibility that other direct or indirect protein-protein binding events could contribute to the interaction between Bmx and Cas. For example, FAK and Src kinases could function in some situations as bridge molecules between Bmx and Cas as they bind to both of these molecules (15, 16, 21, 37, 51).

Expression of Bmx leads not only to a complex formation between Bmx and Cas but also to an enhanced tyrosine phosphorylation of Cas in the SD domain. Previous studies have identified this domain as a direct or indirect target for several kinases including FAK, Src, and Abl that regulate cell motility

30 min. The cells were fixed, permeabilized, and stained with anti-HA and/or anti-Cas antibodies as well as with rhodamine-phalloidin to visualize the actin cytoskeleton. Sheep anti-mouse (Alexa 546) and goat anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate were used to detect the binding of the primary antibodies. B, COS-7 cells were transfected with the indicated plasmids together with pEGFP vector to identify the transfected cells. The cells were serum-starved, detached, and applied in triplicate to Transwells that had been coated on the underside with 10 μ g/ml FN and allowed to migrate for 3 h at 37 °C. The cells in the upper chamber were wiped off, and the cells on the underside of the filter were fixed in 4% paraformaldehyde and counted as described under "Materials and Methods." Expression levels of the transfected proteins are shown by immunoblot analysis in lower panels.

in various cellular models and downstream of a number of different migratory stimuli (reviewed in Refs. 52 and 53). Thus, enhanced tyrosine phosphorylation of the SD domain of Cas appears to invariably coincide with cell motility, suggesting a utility for anti-phospho-Cas antibodies as a putative clinical marker for cell motility and invasiveness. Chen *et al.* (37) have proposed a model in which the interaction between the pleckstrin homology domain of Bmx and the FERM domain of FAK will open up the "closed" conformation of the inactive Bmx and allow it to be catalytically activated by phosphorylation at Tyr-566 by the FAK-associated Src kinase. It is conceivable that activated Bmx could then directly phosphorylate the SD domain in the associated Cas molecule, a notion that is supported by our finding that active Bmx indeed phosphorylates recombinant CasSD *in vitro*. However, we cannot rule out the possibility that an active Src molecule, which is potentially present in the Bmx-Cas complex, could be the kinase responsible for Cas phosphorylation *in vivo*. Studies on the exact molecular mechanisms that mediate Bmx-induced Cas phosphorylation *in vivo* are complicated because of the fact that Src (and perhaps also FAK) could potentially act both upstream and downstream of Bmx with respect to Cas phosphorylation.

Tyrosine phosphorylation of Cas in the SD region is known to lead to its binding to the adapter Crk (14, 30), and we found that to be the same here. Studies by us and others (30, 31, 48) have demonstrated that the Cas-Crk complex formation is instrumental in regulating actin reorganization and cell motility in a Rac-dependent manner. Rac, which is a member of the Rho family of GTPases, is a critical mediator of cell migration by inducing formation of lamellipodia extensions and membrane ruffles (54). Crk binds to tyrosine-phosphorylated Cas through its SH2 domain while its N-terminal SH3 domain is thought to interact with molecules that connect the Cas-Crk complex to the regulation of the Rac signaling pathways (reviewed in Ref. 55). Most notably, DOCK180 is a Crk-SH3-binding protein that we and others have recently shown to function as a novel type of guanine nucleotide exchange factor for Rac (56, 57). Crk also interacts with Sos, which is known to function as a guanine nucleotide exchange factor for Rac under some conditions and also to induce Rac activation via Ras (58–60). Our recent findings indicate that Crk also regulates Rac signaling independent of the GTP loading of Rac by enhancing the capability of active Rac to couple to its downstream effectors (44). We have found, not so surprisingly, that a dominant-negative form of Rac blocks Bmx-induced lamellipodia formation (data not shown), and studies are currently underway to explore the molecular connection between Bmx and Rac.

Activation of the Cas-Crk pathway leads not only to cell migration but also to regulation of the c-Jun N-terminal kinase cascade and c-Jun-dependent gene transcription, cell survival, and cell cycle progression (35, 48, 61, 62). Thus, the role of Cas in mediating additional known cellular effects downstream of Bmx in both integrin- and non-integrin signaling pathways remains to be determined. Likewise, Bmx has been recently reported to directly interact with and activate the serine/threonine kinase PAK1 (63) and the small GTPase RhoA (64) and both of these molecules are known to be integral components of integrin signaling (reviewed in Ref. 65). Further studies are therefore required to fully explore the significance of Bmx in signaling pathways emanating from cell-extracellular matrix interactions.

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